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A novel function of botulinum toxin-associated proteins: HA proteins disrupt intestinal epithelial barrier to increase toxin absorption

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ABSTRACT

Food-borne botulinum neurotoxin (BoNT) in the gastrointestinal lumen must cross an epithelial barrier to reach peripheral nerves to mediate its toxicity. The detailed mechanism by which BoNT traverses this barrier remains unclear.
We found that hemagglutinin (HA) proteins of type B BoNT complex play an important role in the intestinal absorption of BoNT, disrupting the paracellular barrier of intestinal epithelium, which facilitates transepithelial delivery of BoNT both *in vitro* and *in vivo* (Matsumura, T., et al., 2008. Cell. Microbiol. 10, 355–364). We also found that type A HA proteins have a similar disrupting activity with a greater potency than type B HA proteins in the human intestinal epithelial cell lines Caco-2 and T84. In contrast, type C HA proteins in the toxin complex (up to 300 nM) have no detectable effect on the paracellular barrier in these human cell lines. These results may indicate that types A and B HA contribute to develop the food-borne human botulism by facilitating the intestinal transepithelial delivery of BoNTs.

1. Introduction

Botulinum neurotoxin (BoNT, types A ~ G) is a potent, large protein toxin (~150 kDa) that specifically binds to neuronal cells. Upon entering the cytoplasm, the metalloproteinase activity of BoNT cleaves the proteins involved in vesicular fusion thereby inhibiting neurotransmitter release and leading to paralysis (Schiavo et al., 2000). Of the seven serotypes, types A, B, and to a minor extent, E, and F are typically associated with botulism in humans, whereas types C and D mostly affect domestic animals. Food-borne BoNT in the gastrointestinal (GI) lumen must cross an epithelial barrier to reach the peripheral nerves to mediate its toxicity. The mechanism by which BoNT traverses the epithelium and enters the circulation remains unknown, and we wished to address this issue.

BoNT is produced by *Clostridium botulinum* as a non-covalent assembly with other proteins termed NAPs (non-toxic neurotoxin-associated proteins). The BoNT-NAP complex forms either as a 12S or 16S multimeric protein particle (Fujinaga, 2006; Oguma et al., 1999; Sakaguchi et al., 1984) (Fig. 1). The 12S toxin complex contains BoNT and a non-toxic component lacking hemagglutinin (HA) activity, which is designated non-toxic non-HA (NTNH). The 16S toxin complex consists of BoNT assembled with NTNH and three other proteins termed HA1, HA2, and HA3 that together exhibit hemagglutinin (HA) activity. The NTNH and HA proteins make a stable assembly in the absence of BoNT and this protein complex is termed NAP-16.

The NAPs act to enhance BoNT toxicity following oral administration by protecting BoNT from denaturation and degradation in the GI tract (Sakaguchi et al., 1984). We have also found that the HA component binds to the microvilli of the small intestine (Fujinaga, 2006), and this appears to induce 16S toxin internalization into the enterocyte (Uotsu et al., 2006). But whether HA is required for BoNT to breach the intestinal epithelial barrier remains unclear. *In vitro*, purified BoNT in the absence of the HA proteins can pass through a model intestinal epithelial monolayer by

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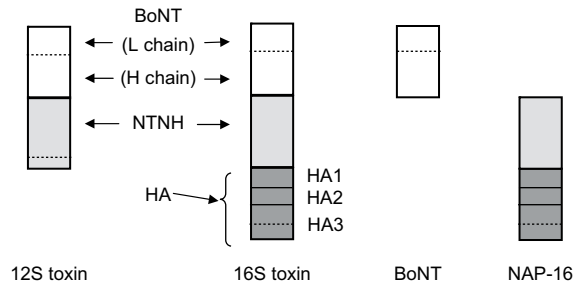

Fig. 1. A schematic representation of type B botulinum neurotoxin complexes and their components.

transcytosis (Couesnon et al., 2008; Maksymowych and Simpson, 1998).

In the current study, we examined the effect of neurotoxin complexes and their constituent parts on human intestinal epithelial cell lines, Caco-2 and T84, and a canine kidney epithelial cell line, MDCK I. These cells were grown on permeable supports as simple columnar epithelia with high transepithelial resistance against the passive diffusion of ion and protein solutes. Our results show an unexpected and potent activity of the HA proteins that disrupts the intercellular junctions of the intestinal epithelium so as to allow passive protein transport via the paracellular pathway.

2. Materials and methods

2.1. Bacterial strains

C. botulinum type A strain 62A supplied by Dr. S. Kozaki (Osaka Prefecture University, Osaka, Japan), as well as type B strain Lamanna and type C strain Stockholm supplied by Dr. K. Oguma (Okayama University, Okayama, Japan) were used in this study.

2.2. Preparation of toxins and non-toxic components

Type B 16S toxin, 12S toxin, and BoNT were purified as described previously (Arimitsu et al., 2003). Type A toxins were purified as described previously (Arimitsu et al., 2003) with some modifications. Type C toxins were purified by a procedure described previously (Fujinaga et al., 1997; Inoue et al., 1999; Mahmut et al., 2002) with minor modifications. The concentrations of the toxin complexes in molarity were calculated from the molecular masses of 16S toxin (745 kDa) and 12S toxin (287 kDa) for the type A HA-positive toxin complex and the HA-negative toxin complex; the molecular weights of 16S toxin (744 kDa) and 12S toxin (289 kDa) for the type B HA-positive and HA-negative toxin complexes; and the molecular weights of 16S toxin (749 kDa) and 12S toxin (287 kDa) for the type C HA-positive and HA-negative toxin complexes, respectively. The molecular mass of each toxin was calculated based on the stoichiometry of subunits contained in the type D toxin complexes (Hasegawa et al., 2007). In the case of type A HA-positive toxin complex (a mixture of 16S and 19S toxins), one molecule of 19S toxin was regarded as equal to two molecules of 16S toxins, and the concentrations of the toxin complex in molarity were calculated as those of 16S toxins.

2.3. Cell culture

Caco-2, T84, and MDCK I cells were plated and cultured onto transwell chambers (Costar) as described previously (Matsumura et al., 2008) with some modifications.

2.4. Measurement of the transepithelial electrical resistance (TER) and the paracellular tracer flux assay

The transepithelial electrical resistance (TER) and the paracellular flux of FITC-dextrans was assessed as previously described (Matsumura et al., 2008).

3. Results and discussion

3.1. Type B HA reduces transepithelial electrical resistance (TER)

The addition of the type B 16S toxin to the apical side of the Caco-2 monolayers caused a time-dependent decrease in transepithelial electric resistance (TER), and a dramatic decrease in TER was seen within 48 h of treatment (Fig. 2A). Neither the purified 12S toxin nor BoNT alone affected TER (data not shown), indicating the requirement of HA action. When added basolaterally, the 16S toxin caused identical effects on TER, but the activity was much more pronounced due to a more rapid onset of the effect (complete loss of TER within a few hours, Fig. 2B) and the greater potency of 16S toxin action.

To confirm that the HA component is required and sufficient for inhibition of epithelial barrier function, we incubated type B 16S toxins with rabbit antisera specific for type B HA or BoNT before the application of the toxin to the monolayers (Matsumura et al., 2008). Pre-incubation of the type B 16S toxin with antibodies against the HA subunits, completely inhibited its activity. In contrast, pre-incubation of the type B 16S toxin with antibodies against the BoNT subunits had no effect. These results show that the HA proteins contained in the type B 16S toxin mediates the inhibitory effect on TER.

3.2. Type B HA disrupts the epithelial paracellular barrier

A reduction in TER is typically associated with disruption of the paracellular barrier, and we tested for type B 16S toxin effects on the paracellular barrier function by an assay for the transepithelial transport of fluorescein-labeled dextrans (Fig. 3). Dextrans do not engage with any known active transport mechanisms in the intestine and the bulk of flux across intact monolayers can be accounted for almost exclusively by passive diffusion across tight junctions and through the paracellular space. The results show that the type B 16S toxin increased paracellular flux of dextrans of all sizes (4 K, 10 K, and 150 K) from apical to basolateral reservoirs, consistent with an inhibitory effect on epithelial barrier function. Pre-incubation of the 16S toxin with the anti-HA antiserum completely reversed this